9 Patient-Like Orthotopic Metastatic Models of Human Cancer

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Contents

1. INTRODUCTION

In 1889 Paget formulated the seed and soil hypothesis of cancer metastasis. Since that time there have ben major efforts to develop animal models of cancer to test and investigate the hypothesis of Paget as well as for treatment and drug discovery. Thus, there has been a critical need in cancer treatment and research for rodent models that are clinically relevant. Ideal models would allow the transplantation of the majority of human tumors such that the tumor would behave in the rodent in a similar manner as it did in the patient. Such models would be useful for individual patient treatment design and for evaluation of new antineoplastic agents and procedures. This review traces the development of rodent animal models from the first transplantation experiments over 100 years ago to the present era. The present era is one of great promise, with the use of immunodeficient rodents, which accept foreign tissues for human tumor transplantation. The modern era is characterized by new orthotopic transplant methodologies that allow human tumors to express their metastatic potential, especially the models developed in our laboratory that

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are constructed by surgical orthotopic implantation with intact tumor tissue. The use of tumors expressing green fluorescent protein and other fluorescent proteins for external whole-body imaging has enabled imaging of tumor growth and metastasis even on internal organs. The SOI models have allowed experimental confirmation of Paget's seed and soil hypothesis of 1889. The use of SOI models for discovery of antimetastatic and antiangiogenesis agents is discussed. The exciting possibility of the use of the SOI models for discovery of antitumor and antimetastatic genes is also discussed.

2. EARLY STUDIES OF TUMOR TRANSPLANTATION

Early studies of transplantation of human tumors to animals ended in failure, including attempts to transplant human tumors to higher primates *(1)*. Ewing *(1)* has described the following historical developments: For example, Sticker tried to transplant a dog lymphosarcoma to foxes in 1904 *(2)*. Experiments such as these had limited success. Transplantation of sarcoma was attempted between dogs by Novinsky in 1876, who had only two successes in 42 attempts *(3)*. Hanau in 1889 had greater success in transplanting a vulva epidermoid carcinoma from a rat to other rats *(4)*. The first systematic study of transplanted tumors was that of Morau in 1894, who transplanted a cylindrical-cell carcinoma of a mouse for a number of generations *(5)*. Loeb in 1901 and 1902 in the United States passaged a cystic sarcoma of the thyroid between rats for 40 generations with maintenance of structure, but no metastases were observed *(6,7)*. Jensen in 1902 and 1903 passaged a mouse sarcoma through 19 generations of mice, again without noting metastases *(8,9)*. Ewing noted that with regard to therapeutics, transplantable tumors "opened up many new trails which unfortunately have led mostly astray" *(1)*.

With regard to metastases, Bashford et al. *(10)* noted local infiltration and metastases in the Jensen tumor, and Apolant *(11)* showed that infiltrative and metastatic growth most frequently occurred when the tumor invaded dense resisting tissues. Many other tumor types have been described in rodents that have long been extensively transplanted. Rous described three chicken sarcomas that were transplantable and showed that cell-free extracts of the spindle cell sarcoma could serve as the agent to induce further tumors in other animals *(12)*.

It was shown that in animals inoculated with the cell-free "juice," as Ewing described it *(1)*, tumors developed at the point of inoculation but always more slowly than when animals were inoculated with tumor cells themselves. Filtrates from Rous' spindle-cell tumor and condroma gave rise to similar tumors from the tumor in which the filtrates were obtained. We now know that the filtrate contained the Rous sarcoma virus, which is one of the most characterized of all tumor viruses.

Ewing noted that nearly all experiments found difficulty in getting the original tumor to engraft in another animal, but subsequent passages usually occurred at higher rates and some tumors such as the buffalo rat sarcoma have eventually been able to be passed at a 100% take rate *(1)*. Ehrlich proposed that tumors, after passage, became increasingly virulent *(13)*. As Ewing noted, among the passaged tumors, the subcutaneous tissue was the site ordinarily used for transplantation. However, internal organs, especially the spleen, have been used for implantation but have proved, according to Ewing, less susceptible *(1)*.

In the early transplantation experiments, it was found that not only was the same species of animal required in transplantation of tumor from one animal to another, but the results were more successful between animals of exactly the same color and antecedent as Ewing described *(1)*. This is owing, of course, to what we now know to be the rejection of transplants from nongenetically identical animals. Ewing noted that Beebe and Van Alstyne *(14)* observed that a carbohydrate-free diet made buffalo rats highly refractory to the buffalo sarcoma and that even the progression of this tumor was retarded by carbohydrate-free diets but was accelerated by butyrates.

Ewing *(1)* stated that many tumors maintained a uniform histology through many generations but that some carcinomas changed greatly through passaging. He also noted that the most striking change induced by the passageable tumors was the induction of malignant sarcoma properties in the normal host stroma. This was first observed by Ehrlich *(13)* and Apoland *(11)* in the 10th generation of passage of a mouse adenocarcinoma. Both the passaged tumor and a now-malignant stroma could persist together or as separate strains of sarcoma and carcinoma. The rate of growth of mixed tumors appears to be enhanced. It was concluded that the sarcoma was caused by a neoplastic transformation of the stroma of the host induced by the stimulation of the stromal cells by the malignant epithelium *(11,13)*. Ewing *(1)* noted another possibility, that the spindle-like cells of the stroma might be in reality altered epithelium; it had been shown repeatedly that animals resisting engraftment of passaged tumors may often develop spontaneous tumors (and vice versa), so Ewing concluded that the problem of continued growth of an engrafted tumor is quite different from "spontaneous" tumor development.

As mentioned above, with regard to heterotransplantation in the early days, it was difficult to find tumors that could be consistently transplanted between different strains of even the same species. The Ehrlich tumor in mice, however, was found to be readily transplantable. The Ehrlich tumor was originally a mammary tumor that transformed into the ascites form and could be transplanted into outbred Swiss mice by even a few cells *(15)*. Researchers then tried to advance heterotransplantation by finding what we now consider potentially immunologically privileged sites in the animal such as the anterior chamber of the eye, the cheek pouch, and the brain, all of which had many limitations *(15)*. Greene *(16)*, for example, used the eye of rabbits and guinea pigs to grow human tumors. as mentioned above,

3. USE OF IMMUNODEFICIENT RODENTS FOR TUMOR TRANSPLANTATION

Another approach to tumor heterotransplantation has been the use of immunoincompetent animals. For example, tumors have been successfully grown in newborn mice and fetuses, which are naturally immunoincompetent *(17,18)*. The fetuses and the newborns, however, become immunocompetent and eventually can reject the transplanted tumor.

Attempts were then made to immunosuppress adult animals such as with whole body radiation and steroids *(19,20)*. This treatment, however, is very damaging to the animal. As Giovanella and Fogh *(15)* point out, the observations that the thymus is critical in rejection allowed heterotransplantation studies to proceed at a faster rate using, for

example, thymectomy or antilymphocyte (ALS) or antithymocyte serum (ATS) *(21–26)*. Giovanella and Fogh *(15)* noted that the most effective combination treatment for immunosuppression of animals to be used as hosts for tumor passaging is thymectomy at birth with total body radiation and later a reconstitution with syngeneic bone marrow *(25,27,28)*. All these procedures are difficult, however, and involve serious side effects.

4. USE OF ATHYMIC NUDE MICE FOR TUMOR HETEROTRANSPLANTATION

4.1. Subcutaneous Implant Tumor Models

A new era was opened with the isolation and identification of genetically immunosuppressed animals. In particular, in 1966 Flannigan *(29)* identified the nude mouse mutant. In 1968, Pantelouris *(30)* showed that this animal was athymic and lacked T-cells and therefore could not reject foreign tissue. If the nude mice are kept in a germ-free environment, the life span can be increased to almost the normal length, thereby making them useful experimental animals. The first reported xenograft in nude mice was a subcutaneously implanted human colon adenocarcinoma *(31)*. in 1972, Giovanella et al. *(32)* inoculated a cell line of a human melanoma in nude mice and obtained an invasive tumor. The most frequently occurring tumors have been successfully xenografted subcutaneously in nude mice *(33)*. Fogh et al. *(34)* successfully subcutaneously xenografted 381 tumors in 6–8-wk-old nude mice from 14 categories of cancer. With regard to take rate they found that the melanomas and colon tumors had the highest take ratio, with breast and lymphoreticular tumors having the lowest. The percentage of takes for the total series was 28%. The take rate was 50% for recurrent tumors, 38% for metastases, and 21% for primary site tumors, *(34)*. One-third of the transplants could be established as lines. If the xenografts could be carried beyond passage three, they had a 90% probability of becoming established as lines. Fogh et al. *(34)* noted that there was a variability in the growth rate of the transplanted tumors in nude mice. Gynecological tumors and colon tumors grew rapidly, and germ cell and bone tumors grew slowly. Metastases and recurrent tumors were found to grow faster than tumors from primary sites, and the less differentiated tumors were more easily established as lines and grew faster *(15)*. Giovanella et al. *(32)* used cultured cells for the first time to initiate tumors in nude mice. This approach was used to assay the tumorigenicity of the cell lines *(35)*. However, it became apparent that some highly malignant-appearing cultured lines formed tumors at a low rate after transplantation into nude mice *(15)*.

Sharkey et al. *(36)* reported that about one-third of the tumors studied in their series increased their differentiation status after growing as xenografts in nude mice. It was also shown that in some human malignant glial tumors implanted in the brains of nude mice, new histological patterns occurred *(37)*. A similar observation was made in a study of 12 human melanomas grown in nude mice *(38)*. Pancreatic adenocarcinomas in nude mice produced many of the pancreatic-specific enzymes, but digestive enzymes were not being produced *(39)*.

In a large series of subcutaneous implantation of surgical specimens Shimosato et al. *(40)* obtained 58 tumor takes out of 243 attempts. Most difficult to implant successfully were breast carcinomas. Rae-Venter and Reid *(41)* have shown that subcutaneous growth of a number of human breast tumors was enhanced by implantation of estrogen. Shafie

and Grantham *(42)* have demonstrated with the MCF-7 human breast carcinoma cell line a lack of growth in animals without functional ovaries and a pancreas, the results of which could be reversed by estrogen and insulin.

A number of models for prostate cancer have been developed including the Dunning R-3327 hormonally dependent rat prostatic adenocarcinoma *(43)*. A number of cell lines from human prostatic carcinoma that grow in athymic nude mice, including the LNCaP line, which is androgen-dependent *(44)*, and the androgen-independent cell lines Du145 *(45)* and PC-3 *(46,47)*, have also been isolated. The usual mode of in vivo growth of the human prostate carcinoma lines has been after subcutaneous transplantation. The use of extracellular matrix proteins such as Matrigel seems to enhance the take rate of subcutaneously implanted tumors *(48)*. However, intrasplenic injection *(49)* has also been used, which may result in more metastatic activity. The PC-3 line has been injected into the tail vein of nude mice while the inferior vena cava was occluded, which allowed tumor growth in the lumbar vertebrae, pelvis, and femur *(50)*. When PC-3 cells were injected into the peritoneal cavity, intra-abdominal growth resulted *(51)*. When they were injected into the spleen, liver metastases resulted, and when they were injected into the seminal vesicles, large tumors developed there *(51)*. However, none of these models is representative of the clinical course of growth of prostate carcinoma and are not of use for predicting the clinical course or response to treatment of individual patients. Shroeder *(52)* implanted flat pieces of prostate tumor directly into exposed subcutaneous muscle where the muscular fascia was scraped away. The PCA-2 human prostrate carcinoma has been serially transplanted in male nude mice but would not grow tumors in female nude mice and regressed after castration or estrogen treatment of the male, thereby demonstrating its hormone dependency *(53)*.

Bullard and Bigger *(54)* and Shapiro et al. *(55)* were successful in growing craniopharyngiomas, glioblastomas, and astrocytomas directly in the brain or in subcutaneous tissue. Schackert et al. *(56)* found that carcinomas of the colon, breast, kidney, and lung, when injected into the cerebrum carotid arteries, produced brain tumors.

A number of investigators have been quite successful in growing bladder carcinoma subcutaneously in nude mice. Sufrin et al. *(57)* succeeded in growing 8 of 20 human transitional cell carcinomas, and Naito et al. *(58)* successfully implanted 8 of 31 human urinary cancers. All these studies were done at the subcutaneous site in the nude mice. Mattern et al. *(59)* were able to implant and passage 22 human lung tumors. Many other human tumor types have now been grown subcutaneously in nude mice including a wide range of sarcomas, lymphomas, leukemias, testicular carcinomas, mesotheliomas, hepatomas, pancreatic carcinomas, and squamous cell carcinomas of the head and neck *(15)*.

Giovanella et al. *(60)* have found that malignancies of many cell types may be expressed only in nude mice that have been further immunosuppressed, for example by ALS or X-ray irradiation. This has been shown for human bladder carcinomas and a human osteosarcoma, which in control nude mice did not form tumors but grew after subcutaneous implantation in X-ray-treated mice. Two-week-old mice have also been proved to be better than 6–8-wk-old mice, since the younger mice are more immunoincompetent. The young mice have allowed certain tumors to grow that could not grow in older animals, such as tumors of the bladder and thyroid and glioblastomas.

With regard to enhancement of tumor growth in nude mice, it has recently been shown that Matrigel significantly accelerated small cell lung carcinoma cell line growth after the

cells and Matrigel were coinjected subcutaneously in nude mice *(61)*. Other human and murine tumor types were found to have their growth stimulated by coinjection of the cells with Matrigel *(62)*.

Sharkey and Fogh *(63)* have shown that it is necessary to demonstrate that the tumors growing in nude mice are of human origin. It has been demonstrated by Giovanella and Fogh *(15)* that the nude mouse stromal elements can be converted by xenografted carcinomas to malignant sarcoma-producing nude mouse tumors, as mentioned above in early studies described by Ewing *(1)*.

A number of more recent studies have shown that the implantation of tumors but not tumor cell lines from humans induced a transformation of murine stromal cells. For example, the transformed stromal cells, after short-term culture in vitro, could produce sarcomas in other nude mice *(64,65)*.

Studies have been done with human premalignant tissue, but such tissue has been difficult to xenograft successfully *(15)*. However, Bhargava and Lipkin 1981 *(66)* were able to demonstrate that benign polyps of the colon could survive for up to 28 d implanted in kidney capsules in nude mice. Normal tissue from fetuses can be implanted in nude mice *(67)*, and human adult tissue can be quite normal after implantation in nude mice *(68,69)*.

Metastases have not been observed in most tumor transplantation experiments in nude mice using the subcutaneous site. However, with regard to melanomas, when the primary tumor was surgically resected, the animals could live longer and could subsequently develop distant metastases, sometimes 7–12 mo after the tumor inoculations *(70)*. Three amelanotic human melanomas cell lines, when injected intradermally, produced metastases in the regional lymph nodes and in one case lymphatics in the lungs *(71)*. Recent studies with another amelanotic melanoma cell line termed LOX *(72,73)*, as well as pigmented melanoma cell lines, have also demonstrated growth and metastasis after subcutaneous or subdermal implantation. Recently, Van Muijen et al. *(74)* showed that by first passaging a human melanoma three times in nude mice and then establishing a cell line, subcutaneous implantation of the cell line caused 90% of the nude mice to demonstrate lung metastases. In the series of Sharkey and Fogh *(75)*, 106 malignant human tumor cell lines were xenografted to 1045 nude mice. Metastases were observed in only 14 animals, involving 11 different tumor lines. Breast tumor cell lines metastasized at the highest frequency, but none of the sarcoma lines metastasized, which was quite different from the human patient situation. These investigators found that deep penetration of the body wall during tumor growth correlated with the occurrence of metastases.

The human tumor lines in the study of Sharkey and Fogh *(75)* included carcinomas in the breast, lung, and gastrointestinal and urogenital tracts, as well as tumors of unknown primary sites. Metastatic sites included local lymph nodes in two cases, distant lymph nodes in two cases, and a spleen and mediastinum in one case each. Metastatic and non-metastatic tumors from the patient population metastasized equally poorly in the nude mice in this series *(15)*. A renal cell adenocarcinoma in some instances metastasized to axillary and inguinal lymph nodes after subcutaneous inoculation *(76)*. The SW480 cultured colon carcinoma was seen to metastasize to the regional lymph nodes after

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subcutaneous implantation, and when the tumor was implanted intraperitoneally, metastasis to the lymph nodes and lungs was seen *(77)*.

Shimosato et al. *(40)* found that after subcutaneous inoculation, an acute lymphocytic leukemia as well as a number of breast cancers metastasized to regional lymph nodes. Kyriazis et al. *(77)* have shown that in addition to the cultured colon carcinoma, transitional cell carcinomas of the bladder and adenocarcinoma of the pancreas could invade and even metastasize to the lymph nodes of the lung after subcutaneous implantation. Neulat-Duga et al. *(78)* found metastasis in 30% of 63 human tumors implanted subcutaneously.

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Therefore, although metastases can occur from subcutaneously growing tumors in nude mice in a number of different tumor types and studies, this is rather rare. Giovanella and Fogh *(15)* emphasize that if the animals bearing the subcutaneously growing tumors can be kept alive for long periods, such as by resecting the primary tumors described above, the probability of metastasis can be raised. Provoked local recurrences at the site of a subcutaneous growth such as reimplantation of tumor fragments or fragments left in the animal following reduction of a tumor load can induce massive lung metastases from xenografted cell lines derived from mammary tumors, colon carcinomas, melanomas, and prostate tumors *(15)*.

Effect of implant site on tumor growth.

4.2. ~~Orthotopic Implants~~

Early studies showed that the site of implantation could influence the growth rate, invasiveness, and metastatic behavior of the resulting tumors. Kyriazis and Kyriazis *(79)* noted that tumor growth in the anterior lateral thoracic wall was faster than in the posterior aspect of the trunk. With regard to human small cell carcinoma, intracranial injection was found to be superior to the subcutaneous or subdermal site. At the intracranial site, takes of 100% with small cell carcinomas were observed by Chambers et al. *(80)*, with one-tenth of those cells required to produce subdermal tumors. The latent time for tumor growth was shorter in the intercranial site than in the subdermal site. Importantly, the tumors grew in the meninges and subsequently invaded and destroyed the brain, in contrast to the subcutaneously growing tumors, which were not invasive. In addition, the MCF-7 cell line grew well in the mammary fat pad, indicating the importance of the orthotopic site *(81,82)* (*see* Subheading 5. below). The mammary fat pad was also shown to be a preferential site for growth and metastasis for estrogen receptor-negative human breast cancer cell lines *(83)*. As mentioned above, the mammary fat pad was shown to be a superior implantation site for tumor growth and metastasis for hormone-independent human breast cancer cells *(42,83)*. As with the small cell carcinoma, the MCF-7 carcinoma was highly invasive after intracranial inoculation *(84)*. When transplanted into the intracranial region, lymphoma and leukemia and cell lines derived even from normal donors produced tumors *(60,85)*. The subrenal capsule site developed by Bogden et al. *(86)* as a means of implanting tumors in nude mice has allowed the growth of many different types of tumors; some of them were shown to be invasive even if they were not implanted at the subcutaneous site. Kyriazis and Kyriazis *(79)* showed that when some tumors were implanted intraperitoneally, the neoplastic growth in the peritoneal cavity was malignant, including the ability to invade various organs by lymphatic and hematogenous routes.

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5. ORTHOTOPIC TRANSPLANT MODELS UTILIZING HUMAN TUMOR TISSUE IN NUDE MICE: *ENHANCED METASTATIC POTENTIAL*

~~Tumor differentiation may be affected by site.~~ For example, Hajdu et al. *(87)* found that subcutaneously growing tumors were better differentiated than the same tumors growing intraperitoneally in nude mice.

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In the last 20 years it has thus become clear that orthotopic sites of implantation are critical to the metastatic capability of the transplanted tumors in nude mice *(88)*. With regard to colon carcinoma, when the cecum and spleen were used as sites of implantation of colon tumor cell lines or disaggregated tumor tissue, metastasis occurred including that to liver as opposed to the subcutaneous site, which allowed primary growth to occur but not metastasis *(89–95)*. Inoculation of tumor cell lines into the descending portion of the large bowel allowed micro- and macroinvasive behavior of the cells via infiltration of the various layers of the mouse colonic wall, in particular the muscularis propria *(89–94)*. This behavior was contrasted to melanoma cell lines implanted into the colon, which exhibited limited invasiveness compared with the colon carcinomas *(94)*.

5.1. Colon Tumor Models

tumor fragments

In our laboratories, histologically intact human colon ~~cancer specimens~~ directly derived surgically from patients were transplanted by surgical orthotopic implantation (SOI) to either the colon or the cecum of nude mice. SOI for colon tumor involves suturing the tumor tissue on the serosa of these organs. We have achieved extensive orthotopic growth in more than 50 cases of patient colon tumors with subsequent regional, lymph node, and liver metastasis, depending on the case. Similar results were found with SOI of intact tissue from a human colon cancer xenograft line, including liver metastasis. Thus, a patient-like model for human colon cancer has been developed that can be used for research into the biology of colon cancer metastases, for the potential prediction of clinical course, for drug response testing of the disease in individual patients, and for the discovery of new therapeutics *(96)*. In a comparison between SOI of intact colon tumor tissue and orthotopic injection of cell suspensions derived from the colon tumor tissue, Furukawa et al. *(97)* observed that SOI performed in intact tissue resulted in high metastatic rates, and the cell suspension injections resulted in no metastases.

Partial hepatectomy has been widely employed in clinical practice as the therapy of choice for primary and metastatic liver tumors. However, the recurrence rate after the treatment remains high, probably because of the growth of residual microscopic lesions. We have observed the effect of partial hepatectomy on the growth of two human colon cancers (Co-3 and AC3603) implanted in the liver of nude mice using SOI. Our results showed a dramatic acceleration of tumor growth following 30% partial hepatectomy, which resembles clinical procedures. Tumor volumes were assessed with calipers on d 15 by abdominal palpation and on d 30 at autopsy by direct measurement. For both Co-3 and AC3603, tumor volumes in the hepatectomized animals were significantly larger than the control at the above two time points ($p < 0.001$). The results demonstrate the stimulating effect of partial hepatectomy directly on the tumor growth in the liver. Furthermore, since conservative partial hepatectomy (30%) is normally used in clinical practice for surgical treatment of liver metastasis, this animal model should be useful for

the clinical investigation of the high recurrence rate of liver metastasis following partial hepatectomy *(98)*.

5.2. Bladder Tumor Models

With regard to bladder carcinomas, Ahlering et al. *(99)* found that two human bladder transitional cell carcinoma lines, when injected transurethrally into the urinary bladders of athymic nude mice, invaded the mouse bladder and metastasized to the lung. Subcutaneous inoculation of these cell lines allowed tumor growth but very little local invasion and no metastases. ~~Recently~~, Theodorescu et al. *(100)* confirmed the results of Ahlering et al. with respect to the RT-4 human bladder carcinoma cell line. Theodorescu et al. found, however, that when a mutated *H-ras* oncogene was transfected into RT-4 such that overexpression of this gene occurred in the selected cell line RT-4-mr-10, the cell line became more invasive after transurethral inoculation. Areas of invasion of transitional cell carcinoma deep into the muscularis propria of the bladder occurred that in some instances extended into the surrounding adipose tissue and vascular spaces. However, no continuous or metastatic spread of RT-4-mr-10 occurred. These findings are in contrast to the effects of subcutaneous injection of these cell lines, which showed no evidence of tissue invasion *(100)*.

In our laboratories, the ras-transfected human bladder RT-4 carcinoma tissue cell line RT-4-mr-10 just described was transplanted by SOI as histologically intact tissue to the nude mouse bladder. Extensive invasive orthotopic growth and local invasion occurred as well as multiorgan metastases in the liver, pancreas, spleen, lung, ovary, kidney, ureter, and lymph nodes. The results for the implanted RT-4-mr-10 cells are in striking contrast to the experiments described above in which RT-4-mr-10 ~~cells~~ injected transurethrally as disaggregated cells exhibited only local invasion and no distant metastasis. These results further indicate the potential of the intact tissue SOI model to allow full expression of the metastatic capacity of human cancer in the nude mouse *(101,102)*. We have ~~recently~~ also demonstrated that the RT-4 parental bladder tumor line is highly metastatic when implanted orthotopically as histologically intact tissue, thereby showing that the *ras* gene had no effect *(103)*.

5.3. Pancreatic Tumor Models

Vezeridis et al. *(104)* reported that the fast-growing variant of the human pancreatic carcinoma COLO 357, when injected as disaggregated cells into the spleen of the nude mice, resulted in metastases to the liver and lungs of the animal. The authors stated, however, that this study bypasses invasion and generates seeding and colonization rather than metastases. A subsequent study was carried out *(105)* using the COLO 357 and L3.3 human pancreatic tumors to compare orthotopic transplantation with subcutaneous inoculation. These authors took tumors that were subcutaneously grown and harvested and sectioned them into 2 × 2 pieces. Xenografts were first attached to the exteriorized pancreas. The pancreas was then wrapped around the xenograft to cover it completely. The edges of the fatty tissue surrounding the pancreas were sutured such that the xenograft would remain covered upon the return of the pancreas to the peritoneal cavity. It was found that most of the animals ~~grew~~ tumors at the orthotopic site of transplantation. Metastasis occurred in the liver, lung, regional lymph nodes, and distant lymph nodes. The authors felt that by using tumor pieces as xenografts rather than injecting tumor cells

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into the pancreas, the probability of injecting tumor cells into the circulation with subsequent seeding and colonization was eliminated. They emphasized that their model was similar to the human situation of pancreatic cancer: the retroperitoneal nodes, liver, and lungs become involved.

Marincola et al. *(106)* also orthotopically implanted human pancreatic cancer cell lines, in their case in the doudenal lobe of the pancreas for comparison with heterotopic implantation at the hepatic and subcutaneous sites. Intrapancreatic tumor growth was occasionally associated with liver metastases in the animals that were killed after 28 d, 17.8% in young animals and 22.2% in adult animals. However, after more than 45 d of tumor growth, the incidence of hepatic metastases increased to 57.1%. Direct extension of the tumor into surrounding tissues was frequently observed, with involvement of the duodenum of 84% in growing tumors, the kidneys in 31%, and other intra-abdominal organs in 44%. Subcutaneously growing tumors did not give rise to ~~detectable~~ metastases.

In our laboratories, we have implanted histologically intact human pancreatic tumor tissue in the pancreas of the nude mouse and have achieved tumor growth in six of six patient cases *(107)*. Extensive local growth occurred in all cases, with regional extension and frequent metastases to lymph nodes and visceral organs *(107)*.

We evaluated the efficacy of mitomycin C (MMC) and 5-fluorouracil (5-FU) against the human pancreatic adenocarcinoma cell line PAN-12-JCK in an SOI human metastatic pancreatic cancer nude mice model. Implantation was in the tail portion of the pancreas near the spleen. The PAN-12-JCK cells grew very aggressively in the control group of nude mice, with extensive local invasion and distant metastases to various organs; a propensity for the lung was seen, but other organs were involved as well, including the liver, kidney, and regional and distant lymph nodes. Remarkably, none of the mice in the MMC-treated group developed tumors. Although mice in the 5-FU-treated group survived statistically significantly longer than those in the untreated control, the overall incidence of metastasis in these mice was equivalent to those in the control group. However, no liver or kidney metastases were found in the 5-FU-treated animals, perhaps accounting in part for their longer survival. This clinical nude mouse model of highly metastatic pancreatic cancer can now be used to discover new effective agents for this disease *(108)*.

After SOI of the human tumor xenograft PAN-12-JCK into the tail of the nude mouse pancreas, MMC and cisplatin (DDP) were administered intraperitoneally at doses of 4 and 6 mg/kg, respectively, on d 7. The mice were observed for 95 d. There was a statistically significant increase in disease-free and overall survival rates in the MMC and MMC + DDP-treated groups. Local tumor growth was eliminated only in the group treated with MMC + DDP. Hepatic metastasis and peritoneal disseminations were completely inhibited by MMC but not DDP. This study demonstrated the usefulness of the SOI model of pancreatic cancer for studying the differential efficacy of agents affecting primary tumor growth metastasis and survival *(109)*.

Two human pancreatic cancer cell lines expressing green fluorescent protein (GFP), MIA-PaCa2 and BxPC-3, were studied in SOI models. BxPC-3-GFP tumors developed rapidly in the pancreas and spread regionally to the spleen and retroperitoneum as early as 6 wk. Distant metastases in BxPC-3-GFP lines were rare. In contrast, MIA-PaCa-2-GFP lines grew more slowly in the pancreas but rapidly metastasized to distant sites including liver and portal lymph nodes. Regional metastases in MIA-PaCa-2-GFP lines

were rare. These studies demonstrate that pancreatic cancers have highly specific and individual "seed-soil" interactions governing the chronology and sites of metastatic targeting *(110)*.

Two GFP-expressing pancreatic tumor cell lines, BXPC-3 and MiaPaCa-2, were implanted by SOI as tissue fragments in the body of the pancreas of nude mice. Whole-body optical images visualized real-time primary tumor growth and formation of metastatic lesions that developed in the spleen, bowel, portal lymph nodes, omentum, and liver. Intravital images in the opened animal confirmed the identity of whole-body images. The whole-body images were used for real time quantitative measurement of tumor growth in each of these organs. Intravital imaging was used for quantification of growth of micrometastasis on the liver and stomach. Whole-body imaging was carried out with either a transilluminated epifluorescence microscope or a fluorescence light box, both with a thermoelectrically cooled color CCD camera. The simple, noninvasive, and highly selective imaging made possible by the strong GFP fluorescence allowed detailed simultaneous quantitative imaging of tumor growth and multiple metastasis formation of pancreatic cancer. The GFP imaging affords unprecedented continuous visual monitoring of malignant growth and spread within intact animals without the need for anesthesia, substrate injection, contrast agents, or restraint of animals required by other imaging methods. The GFP imaging technology will facilitate studies of modulators of pancreatic cancer growth including inhibition by potential chemotherapeutic agents *(111)*.

Parathyroid hormone-related protein (PTHrP) is an oncoprotein that regulates the growth and proliferation of many common malignancies including pancreatic cancer. Previous studies have shown that PTHrP is produced by human pancreatic cancer cell lines, can be seen in the cytoplasm and nucleus of paraffin-embedded pancreatic adenocarcinoma tumor specimens, and is secreted into the media of cultured pancreatic adenocarcinoma cells. We hypothesized that PTHrP could serve as a tumor marker for the growth of pancreatic cancer in vivo. To test this hypothesis, we used the SOI model of the human pancreatic cancer line BxPC-3. This tumor was stably transduced with GFP to facilitate visualization of tumor growth and metastases. At early (5 wk) and late (13 wk) time points after SOI, serum PTHrP was measured, and primary and metastatic tumor burden was determined for each mouse by GFP expression. By 5 wk after SOI (early group), the mean serum PTHrP level was 32.7 pg/mL. In contrast, at 13 wk post SOI (late group), the mean serum PTHrP level increased to 155.8 pg/mL. These differences were highly significant ($p < 0.001$, Student's t-test). Numerous metastatic lesions were readily visualized by GFP in the late group. Serum PTHrP levels measured by immunoassay correlated with primary pancreatic tumor weights ($p < 0.01$). PTHrP levels were not detectable (<21 pg/mL) in any of the 10 control mice with no tumor. Western blotting of BxPC-3-GFP tumor lysates confirmed the presence of PTHrP. BxPC-3-GFP tumor tissue stained with antibody to PTHrP. These results indicate that PTHrP has a high potential as a useful tumor marker for clinical pancreatic adenocarcinoma in the future *(112)*.

italics

5.4. Head and Neck Tumor Models

With regard to head and neck tumors, Dinesman et al. *(113)* implanted 42 nude mice with laryngeal squamous cell carcinoma cell lines on the floor of the mouth. Pulmonary metastases were noted in 44%, bone invasion in 80%, angioinvasion in 76%, and soft

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tissue invasion in 96% of the animals, thereby mimicking the clinical state. In the head and neck study, lymph node metastasis was seen in only 2 of the 42 animals. In comparison, the subcutaneous model of transplantation for head and neck tumors has not resulted in metastases of tumors that did eventually take, which was at a low rate. For example, Brackhuis et al. *(114)* implanted 130 head and neck carcinomas in subcutaneous tissues of nude mice with a 26% take rate and no observed metastases.

Our laboratory has utilized tumor material directly from surgery from human head and neck cancer patients, including metastatic tongue and laryngeal tumors, and implanted them as histologically intact tissue into the muscles of the floor of the mouth including the mylohyoid muscle as further examples of the SOI technique. We have observed subsequent invasions into the structures of the head (X. Fu and R.M. Hoffman, unpublished observations). When the same tumor tissue was implanted subcutaneously, even in the neck area, extensive tumor growth occurred without subsequent invasion.

5.5. Stomach Tumor Models

The human gastric cancer cell line G/F was implanted either subcutaneously or into the stomach wall of nude mice *(115)*. The G/F tumor implanted in the stomach wall showed a slower growth rate than when the tumor was implanted subcutaneously. Importantly, the tumor implanted in the stomach wall grew and invaded the surrounding tissues and metastasized to the regional lymph nodes and distant organs such as the lung and liver in 27 of 43 mice. In contrast, the tumors growing subcutaneously were highly encapsulated, and metastasis to other organs was not observed. Thus the stomach wall provided a superior microenvironment for the G/F gastric cancer to express its metastatic properties.

SOI of human stomach cancer tissue fragments derived from cell lines resulted in the formation of metastases in 100% of the mice, with extensive primary growth to the regional lymph nodes, liver, and lung. In contrast, when cell suspensions were used to inject stomach cancer cells at the same site, metastases occurred in only 6.7% of the mice with local tumor formation, emphasizing the importance of using intact tissue to allow full expression of metastatic potential. Injuring the serosa, as ~~occurs~~ occurs in intact tissue transplantation, did not increase the metastatic rate after orthotopic injection of cell suspensions of stomach tumor cells. This intact tissue orthotopic implantation model should allow development of new treatment modalities and further study of the biology of human stomach cancer *(116)*.

Fresh surgical specimens derived from 36 patients with advanced stomach cancer were transplanted in nude mice using SOI. Twenty of 36 patient tumors gave rise to locally growing tumors in the mice. All 20 patients whose stomach tumors resulted in local growth in the nude mice had clinical lymph node involvement, whereas 8 of the other 16 patients whose tumors were rejected had lymph node involvement. There was a statistical correlation ($p < 0.01$) between local tumor growth in nude mice and clinical lymph node involvement. Of the 20 cases resulting in local growth in the nude mice, 5 had clinical liver metastases and all 5 cases resulted in liver metastases in the nude mice. Of the 20 cases, 6 had clinical peritoneal involvement of their tumor; of these, 5 resulted in peritoneal metastasis in the nude mice. There were statistical correlations ($p < 0.01$) for both liver metastases and peritoneal involvement between patients and mice. These results indicate that, after orthotopic transplantation of histologically intact stomach

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Of the 15 patients without liver metastases whose primary tumor grew locally in the mice, only one case gave rise to a liver metastasis in a mouse.

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cancers from patients to nude mice, the subsequent metastatic behavior of the tumors in the mice closely correlated with the course of the tumors in the patients *(117)*.

GFP gene was administered to intraperitoneally growing human stomach cancers in nude mice to visualize future regional and distant metastases. GFP retroviral supernatants were injected ip from d 4 to d 10 following ip implantation of the cancer cells. Tumor and metastasis fluorescence was visualized every other week with the use of fluorescence optics via a laparotomy on the tumor-bearing animals. Two weeks after retroviral GFP delivery, GFP-expressing tumor cells were observed in gonadal fat, greater omentum, and intestine, indicating that these primary intraperitoneally growing tumors were efficiently transduced by the GFP gene and could be visualized by its expression. At the second and third laparotomies, GFP-expressing tumor cells were found to have spread to lymph nodes in the mesentery. At the fourth laparotomy, widespread tumor growth was observed. No normal tissues were found to be transduced by the GFP retrovirus. Thus, reporter gene transduction of the primary tumor allowed detection of its subsequent metastasis. This gene therapy model could be applied to primary tumors before resection or other treatment for a fluorescence early detection system for metastasis and recurrence *(118)*.

The efficacy of a (phosphorothioate) antisense oligonucleotide for KDR/Flk-1 (KDR/Flk-1-ASO), an endothelial cell-specific vascular endothelial growth factor (VEGF) receptor, was investigated ~~to reveal~~ the metastasis dissemination and angiogenesis of a human gastric cancer cell line in nude mice. GFP-transduced NUGC-4 (NUGC-4-GFP) human gastric cancer cells were implanted into the peritoneal cavity of nude mice. KDR/Flk-1-ASO, -SO, or PBS were administered from d 7 to d 14, 200 μg/mouse, once a day. The mice were sacrificed on d 28. Disseminated peritoneal tumor nodules expressing GFP were visualized by fluorescence microscopy. KDR/Flk-1-ASO significantly decreased the extent of peritoneal dissemination of the tumors. The number of cells undergoing apoptosis was significantly increased in the KDR/Flk-1-ASO-treated tumors. Microvessel density (MVD) was significantly reduced in the KDR/Flk-1-ASO-treated tumor nodules. The KDR/Flk-1 antisense strategy, therefore, decreases tumor dissemination apparently by inhibiting angiogenesis *(119)*.

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5.6. Lung Tumor Models

Recent studies have demonstrated that inoculation of human lung tumor cell lines intrathoracically or intrabronchially into nude mice *(120,121)* results in orthotopic growth.

~~The MetaMouse approach utilizing histologically intact tumor tissue was applied to~~ lung tumor implantation in the left lung in nude and SCID mice by a thoracotomy procedure we have developed. Results thus far *(122)* indicate that this method not only allows extensive local growth in nude and SCID mice but also allows development of regional and distant metastases as described above.

SOI was used for

When a poorly differentiated large cell squamous cell patient tumor was transplanted orthotopically to the left lung as histologically intact tissue directly from surgery, five of five mice produced locally grown tumors. Opposite lung metastases occurred, as well as lymph node metastases. When grown subcutaneously, this tumor grew locally, but no metastases were found *(122)*.

When the human small cell lung carcinoma cell line Lu-24 was transplanted histologically intact into the left lung of nude mice via thoracotomy after harvesting of subcuta-

neously growing tissue from nude mice, five of five mice produced locally growing tumors averaging 10 mm in diameter within 24 d. All five mice produced regional metastases including tumor invasion of the mediastinum, the chest wall, and the pericardium and distant metastases including the right lung, esophagus, diaphragm, parietal pleura, and lymph nodes. These five mice were implanted with only one 1.5-mm^3 piece of tissue. Three SCID mice were also implanted orthotopically with histologically intact Lu-24 tissue via thoracotomy. All three animals produced locally growing tumors averaging 7.5 mm in diameter within 17 d. All three SCID mice also developed regional metastases including the mediastinum, left chest wall, and pericardium and distant metastases including the opposite lung, lymph nodes, parietal pleura, and diaphragm. The time when symptoms could be observed in the nude mice after transplantation of Lu-24 via thoracotomy was 24 d, as mentioned above; in the SCID mice, it was only 17 d, with the tumor seemingly growing and metastasizing more rapidly in the SCID mice *(122)*.

Similar results were found after orthotopically transplanting histologically intact tissue of human small cell lung carcinomas Lu-130 and H-69; very large local growth and metastases to the opposite lung and distant lymph nodes were seen. These results contrast with the orthotopic injection of a suspension of small cell carcinoma cells in nude rats, resulting in poor local growth and no metastases *(123,124)*.

By implantation of histologically intact human tumor tissue in the parietal or visceral pleura of nude mice, we were able to construct models of early and advanced pleural cancer, respectively. Indeed, symptoms and survival of pleural-implanted mice closely resemble the clinical situation, showing a statistically significant difference in survival between parietal- and visceral-pleural implanted mice, the latter representing an advanced stage cancer. Thus such models, reflecting clinical features, should be of great value in the development of new drugs and treatment strategies *(125)*.

Human malignant pleural mesothelioma is an aggressive cancer with no effective treatment. A relevant animal model is needed for studying the biology and for discovery of effective treatment. To meet this need, we have developed an orthotopic transplant model of human malignant pleura mesothelioma in nude mice that closely mimics the pattern found in the mesothelioma patient. Fresh specimens derived from four patients with malignant mesothelioma were implanted on the parietal pleural of nude mice. All patient tumors gave rise to locally growing tumors in the mice. The transplanted mice presented with symptoms of malignancy such as decrease in physical activity and signs of tumor-related respiratory distress. These animals were shown to have extensive tumor spread in the ipsilateral as well as contralateral pleural cavity and mediastinal lymph nodes. When the lesions were still confined to the ipsilateral parietal pleura, the implanted animals were asymptomatic. The macroscopic features usually found in the patients were also found in the implanted animals such as nodules and masses as well as pleural thickness owing to tumor spread. Histological examination revealed malignant mesothelioma similar to that from which the original tumor specimen was derived. Orthotopic parietal-pleura implantation of fresh histological human malignant mesothelioma thus allows mesothelioma growth in an animal model that very closely mimics the clinical pattern of the human disease. This model provides for the first time a useful human model for biological studies of this disease and for developing effective treatment *(126–129)*.

To understand the skeletal metastatic pattern of non-small cell lung cancer, we developed a stable high-expression GFP transductant of the human lung cancer cell line H460

(H460-GFP). The GFP-expressing lung cancer was visualized to metastasize widely throughout the skeleton when implanted orthotopically in nude mice. H460 was tranduced with the pLEIN retroviral expression vector containing the ~~enhanced~~ GFP (EGFP) and the neomycin (G418) resistance gene. A stable high GFP-expressing clone was selected in vitro using 800 μg/mL G418. Stable high-level expression of GFP was maintained in subcutaneously growing tumors formed after injecting H460-GFP cells in nude mice. To utilize H460-GFP for visualization of metastasis, fragments of subcutaneously growing H460-GFP tumors were implanted by SOI in the left lung of nude mice. Subsequent micrometastases were visualized by GFP fluorescence in the contralateral lung, and plural membrane and widely throughout the skeletal system including the skull, vertebra, femur, tibia, pelvis, and bone marrow of the femur and tibia. The use of GFP-expressing H460 cells transplanted by SOI revealed the extensive metastatic potential of lung cancer in particular to widely disseminated sites throughout the skeleton. This new metastatic model can play a critical role in the study of the mechanism of skeletal and other metastases in lung cancer and in screening of therapeutics that prevent or reverse this process *(130)*.

The Lewis lung carcinoma has been widely used for many important studies. However, the subcutaneous transplant or orthotopic cell suspension injection models have not allowed expression of it's full metastatic potential. A powerful new highly metastatic model of the widely used Lewis lung carcinoma was developed using SOI tumor fragments and enhanced GFP transduction of the tumor cells. To achieve this goal, we first developed in vitro a stable high-expression GFP transductant of the Lewis lung carcinoma with the pLEIN retroviral expression vector containing the ~~enhanced *Aequorea victoria*~~ GFP gene. Stable high-level expression of GFP was maintained in vivo in subcutaneously growing Lewis lung tumors. The in vivo GFP-expressing tumors were harvested and implanted as tissue fragments by SOI in the right lung of additional nude mice. This model resulted in rapid orthotopic growth and extensive metastasis visualized by GFP expression. In all, 100% of the animals had metastases on the ipsilateral diaphragmatic surface, contralateral diaphragmatic surface, contralateral lung parenchema, and mediastinal lymph nodes. Heart metastases were visualized in 40%, and brain metastases were visualized in 30% of the SOI animals. Mice developed signs of respiratory distress between 10 and 15 d post tumor implantation and were sacrificed. The use of GFP-transduced Lewis lung carcinoma transplanted by SOI reveals for the first time the high malignancy of this tumor and provides an important useful model for metastasis, angiogenesis, and therapeutic studies *(131)*.

5.7. Prostate Tumor Models

The human hormone-independent prostate cancer lines Du145 and PC-3 were transplanted into nude mice using SOI. The tumor grew locally and became invasive and metastatic. The tumor invaded the lamina propria of the mouse urinary bladder. The local large tumor growth on the prostate of the mice caused urinary obstruction and hydronephrosis, and local and distal lymph node and lung metastases were observed *(132,133)*.

Intact tissue of the androgen-dependent human prostate cancer cell line LNCaP was implanted on the ventral lateral lobes of the prostate gland by SOI in a series of 20 nude mice. Mice were autopsied, and histopathological examination of primary tumors and relevant organs was performed to identify and quantitate micrometastasis. Eighteen of 20 animals transplanted with LNCaP by SOI had tumor growth. Mean primary tumor weight

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in the prostate was 9.24 g at time of necropsy. Sixty-one percent of the transplanted animals had lymph node metastasis. Forty-four percent had lung metastasis. Mean survival time was 72 d, indicating a high degree of malignancy of the tumor. The extensive and widespread lung metastasis as well as lymph node metastasis following orthotopic implantation of LNCaP in nude mice and the short survival time provide a high-malignancy nude model of the LNCaP human prostate tumor *(134)*.

A fluorescent spontaneous bone metastatic model of human prostate cancer was developed by SOI of GFP-expressing prostate cancer tissue. A high-GFP-expression PC-3 clone was injected subcutaneously in nude mice, and stable high-level expression of GFP was maintained in the growing tumors. To utilize GFP expression for metastasis studies, fragments of the fluorescent subcutaneously growing tumor were implanted by SOI in the prostate of nude mice. Subsequent micrometastases and metastases were visualized by GFP fluorescence throughout the skeleton including the skull, rib, pelvis, femur, and tibia. The central nervous system was also involved with tumor, including the brain and spinal cord as visualized by GFP fluorescence. Systemic organs including the lung, plural membrane, liver, kidney, and adrenal gland also had fluorescent metastases *(135)*.

5.8. Ovarian Tumor Models

Three examples of human ovarian cancer were transplanted by SOI into the ovarian capsule into nude mice in our laboratory. In three cases, we observed three completely different patterns of tumor growth. In the first case, a highly encapsulated tumor developed measuring 33 x 23 mm with watery fluid. No rupture or intraperitoneal seeding was observed. This tumor grew with a cystadenocarcinoma growth pattern. During autopsy, a very small metastatic nodule on the lung of the mouse was observed. In the second case, extensive primary tumor growth was observed. Extensive seeding on the colon and parietal peritoneum of the nude mouse was also found.

The nude mouse models of human ovarian carcinoma described above therefore have the following characteristics:

1. They can be constructed directly from patient tumor specimens.
2. The tumors grow locally in the ovary.
3. The tumors can metastasize to the lung, can seed and grow in the peritoneal wall, and can involve critical organs such as the colon, all of which reflects the clinical situation.

~~The approach to the construction of models of human ovarian cancer described here should eventually be of clinical use *(136)*.~~

An ovarian tumor line (RMG-1: human clear cell carcinoma of the ovary) previously grown subcutaneously was implanted orthotopically as intact tissue into the ovarian capsule of 22 nude mice. The tumors showed progressive growth at the orthotopic site in all animals. The tumor marker tumor-associated serum galactosyltransferase (GAT) tended to be positive in all nude mice. The tumors invaded or metastasized to the contralateral ovary (1/22), retroperitoneum (6/22), mesentery (2/22) and peritoneum (1/22), and omentum (6/22) and metastasized to the subcutaneous tissue (1/22), lymph nodes (9/22), and distant organs including the liver, kidney, pancreas, and diaphragm. In striking contrast, subcutaneous transplantation of this tumor resulted in growth in only

two of five animals, with local lymph node and kidney involvement but no retroperitoneal or peritoneal involvement. These findings suggest that orthotopic implantation provides a suitable microenvironment in which ovarian cancer can express its intrinsic clinically relevant properties. This approach is relevant to the clinical features of ovarian cancer and is thought to be a useful model for studies of therapy for this cancer *(137)*.

5.9. Breast Tumor Models

Histologically intact patient breast tumor tissue was transplanted as intact tissue to the mammary fat pad of nude mice where the tumor tissue grew extensively and metastasized to the lung. ~~This is the first orthotopic transplant metastatic model of human breast cancer *(138)*.~~

We developed an optically imageable orthotopic metastatic nude mouse model of the human breast cancer line MDA-MB-435 expressing GFP. We have demonstrated fluorescent imaging of primary and metastatic growth in live tissue and in intact animals. Fragments of tumor tissue expressing GFP were sutured into the pocket in the right second mammary gland using SOI. Tumor tissue was strongly fluorescent, allowing whole-body imaging of tumor growth by wk 5. Neovascularization of the primary tumor was also visualized by whole-body imaging by contrast of the vessels to the fluorescent tumor. At autopsy, MDA-MB-435-GFP cells were found to have metastasized to various organs, including the lung in ~~55~~%, the lymph nodes in ~~15~~% (including axillary nodes), and the liver in 10% of the animals. These metastases could be visualized in fresh tissue by fluorescence imaging. Detailed fluorescence analysis visualized extensive metastasis in the thoracic cavity and the lymphatic system. Large metastatic nodules in the lung involved most of the pulmonary parenchyma in all lobes. Lymph node metastasis was found mainly in the axillary area. In the liver, fluorescent macroscopic metastatic nodules were found under the capsule. The metastatic pattern in the model thus reflected clinical metastatic breast cancer and provides a powerful model for drug discovery for this disease *(139)*.

5.10. GFP Models

Mouse models of metastatic cancer with genetically fluorescent tumor cells that can be imaged in fresh tissue, *in situ* as well as externally, have been developed. These models have opened many new possibilities including real-time tumor progression and metastasis studies on internal organs and real-time drug response evaluations. The GFP gene, cloned from bioluminescent organisms, has now been introduced into a series of human and rodent cancer cell lines in vitro to express GFP stably in vivo after transplantation to metastatic rodent models. Techniques were also developed for transduction of tumors by GFP in vivo. With this fluorescent tool, tumors and metastasis in host organs can be imaged down to the single cell level. GFP tumors on the colon, prostate, breast, brain, liver, lymph nodes, lung, pancreas, bone, and other organs have also been visualized externally (transcutaneously by quantitative whole-body fluorescence imaging). Real-time angiogenesis has also been imaged and quantified using GFP technology. The GFP technology allows a fundamental advance in the visualization of tumor growth and metastasis in real time in vivo *(140–~~189~~)*.

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6. POSSIBLE MECHANISMS OF ENHANCED METASTASIS OF HETERO-TRANSPLANTED TUMORS AT THE ORTHOTOPIC SITE

As to why orthotopically growing tumors metastasize to a much greater extent than ectopically growing tumors, Reid and Zvibel ~~(181)~~ indicated that investigators have identified a number of parameters: (1) anatomy, which determines the local microenvironment including the location of an available capillary bed; (2) formation of tumor emboli; (3) molecules that specify attachment of tumor cells to particular cells or to extracellular matrix molecules that are tissue-specific; (4) local growth factors; and (5) local matrix chemistries.

Colon tumor cells implanted intracecally grew and produced metastases and also produced higher levels of heparinase and 92- and 64-kDa species of type IV collagenase than the same tumors planted subcutaneously, which did not metastasize ~~(182)~~. Nakajima et al. ~~(183)~~ speculate that these enzymes are critical for the enhanced metastatic capability of the orthotopically implanted tumor.

Leighton ~~(185)~~ postulated that fibroblasts can orient and mediate the directionality of cell spread in metastases. Thus, the stromal elements at the orthotopic site such as fibroblasts might be more interactive with the organ-specific tumor epithelium, with regard to directing cellular invasion, than at ectopic sites.

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7. EXPERIMENTAL PROOF OF PAGET'S SEED AND SOIL HYPOTHESIS WITH METAMOUSE MODELS

Tumors that metastasize do so to preferred target organs. More than 100 years ago, Paget formulated his seed and soil hypothesis to explain this apparent specificity, i.e., the cells from a given tumor would "seed" only favorable "soil" offered by certain groups. The hypothesis implies that cancer cells must find a suitable "soil" in a target organ, i.e., one that supports colonization, for metastasis to occur. The ability of human colon cancer cells to colonize liver tissue governs whether a particular colon cancer is metastatic. In the model used in this study, human colon tumors are transplanted into the nude mouse colon as intact tissue ~~blocks~~ by SOI. These implanted tumors closely simulate the metastatic behavior of the original human patient tumor and are clearly metastatic or nonmetastatic to the liver. Both classes of tumors are equally invasive locally into tissues and blood vessels. However, the cells from each class of tumor behave very differently when directly injected into nude mouse livers. Only cells from metastasizing tumors are competent to colonize after direct intrahepatic injection. Also, tissue ~~blocks~~ from metastatic tumors affixed directly to the liver resulted in colonization, whereas no colonization resulted from nonmetastatic tumor tissue ~~blocks~~ even though some growth occurred within the tissue ~~block~~ itself. Thus, local invasion (injection) and even adhesion to the metastatic target organ ~~(blocks)~~ are not sufficient for metastasis. The results suggest that the ability to colonize the liver is the governing step in the metastasis of human colon cancer *(184)*.

To understand further the role of the host organ in tumor progression, we have transplanted into nude mice histologically intact human colon cancer tissue on the serosal layers of the stomach (heterotopic site) and the serosal layers of the colon (orthotopic site). The xenograft lines Co-3, which is well differentiated, COL-3-JCK, which is ~~and~~ poorly differentiated, were used for transplantation. After orthotopic transplantation of the human colon tumors on the nude mouse colon, the growing colon tumor resulted in

macroscopically extensive invasive local growth in 4 of 10 mice, serosal spreading in 9 of 10 mice, muscularis propria invasion in 1 of 10 mice, submucosal invasion in 3 of 10 mice, mucosal invasion in 3 of 10 mice, lymphatic duct invasion in 4 of 10 mice, regional lymph node metastasis in 4 of 10 mice, and liver metastasis in 1 of 10 mice. In striking contrast, after heterotopic transplantation of the human colon tumor on the nude mouse stomach, a large growing tumor resulted but with only limited invasive growth and without serosal spreading, lymphatic duct invasion, or regional lymph node metastasis. It has become clear from these studies that the orthotopic site, in particular the serosal and subserosal transplant surface, is critical to the growth, spread, and invasive and metastatic capability of the implanted colon tumor in nude mice. These studies suggest that the original host organ plays the same critical role in tumor progression *(185)*.

We have found an exquisite specificity of metastasis in that a metastatic human colon tumor transplanted to the liver of nude mice specifically "reverse metastasized" to the colon of the mouse. The results demonstrate the selective affinity of cancer to the matched soils of the primary and metastatic organs *(186)*.

8. DRUG DISCOVERY WITH PATIENT-LIKE MOUSE MODELS OF CANCER

Matrix metalloproteinases (MMPs) have been implicated in the growth and spread of metastatic tumors. This role was investigated in an orthotopic transplant model of human colon cancer in nude mice using the MMP inhibitor BB-94 (batimastat). Fragments of human colon carcinoma (1–1.5 mm) were surgically implanted orthotopically on the colon in 40 athymic nu/nu mice. Administration of BB-94 or vehicle (phosphate-buffered saline, pH 7.4, containing 0.01% Tween 80) commenced 7 d after tumor implantation (20 animals/group). Animals received 30 mg/kg BB-94 ip once daily for the first 60 d and then three times weekly. Treatment with BB-94 caused a reduction in the median weight of the primary tumor from 293 mg in the control group to 144 mg in the BB-94-treated group ($p < 0.001$). BB-94 treatment also reduced the incidence of local and regional invasion, from 12 of 18 mice in the control group (67%) to 7 of 20 mice in the treated group (35%). Six mice in the control group were also found to have metastases in the liver, lung, peritoneum, abdominal wall, or local lymph nodes. Only two mice in the BB-94 group had evidence of metastatic disease, in both cases confined to the abdominal wall. The reduction in tumor progression observed in the BB-94-treated group translated into an improvement in the survival of this group, from a median survival time of 110 d in the control group to a median survival time of 140 d in the treated group ($p < 0.01$). Treatment with BB-94 was not associated with any obvious toxic effect, and these results suggest that such agents may be effective as adjunctive cancer therapies *(187)*.

CT1746, an orally active synthetic MMP inhibitor, has a greater specificity for gelatinase A, gelatinase B, and stromelysin than for interstitial collagenase and matrilysin. CT1746 was evaluated in a nude mouse model that better mimics the clinical development of human colon cancer. The model is constructed by SOI of the metastatic human colon tumor cell line Co-3. Animals were gavaged with CT1746 twice a day at 100 mg/kg for 5 d after the SOI of Co-3 for 43 d. In this model, CT1746 significantly prolonged the median survival time of the tumor-bearing animals from 51 to 78 d. Significant efficacy of CT1746 was observed on primary tumor growth (32% reduction in mean tumor area at d 36), total spread, and metastasis (6/20 treated animals had no detectable spread and

metastasis at autopsy compared with 100% incidence of ~~secondaries~~ metastasis in control groups). CT1746 was also efficacious in reducing tumor spread and metastasis to individual organ sites such as the abdominal wall, cecum, and lymph nodes compared with vehicle and untreated controls. We conclude that chronic administration of a peptidomimetic MMP inhibitor via the oral route is feasible and results in inhibition of solid tumor growth, spread, and metastasis with increase in survival in this model of human cancer, thus converting aggressive cancer to a more controlled indolent disease *(188)*.

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An SOI model of the human colon cancer cell line Co-3 in nude mice was treated with two doses of the new platinum analogs [Pt(cis-dach)(DPPE) · $2NO_3$] and [Pt(trans-dach)(DPPE) · $2NO_3$]. The analogs were evaluated for antimetastatic efficacy in comparison with two doses of cisplatinum. Unlike the untreated control group, there were no mesenteric lymph node metastases in the groups treated with the high or low doses of both forms of new DPPE platinum analogs as well as the cisplatinum-treated group. However, much more body weight loss occurred in the cisplatinum-treated group than the DPPE-treated groups. The results obtained with the SOI animal model of colon cancer demonstrated that both *cis*- and *trans*-forms of DPPE had as strong an inhibitory effect on metastasis as that of cisplatinum, but with much less toxicity. Thus, the new platinum analogs appears to have promising clinical potential *(189)*.

An SOI model of the human RT-4 bladder tumor in nude mice resulted in local growth, invasion, regional extension, and metastases as well as distant metastases to other organ sites and lymph nodes, thus mimicking the bladder cancer patient. This metastatic bladder tumor animal model was treated with two doses of the new platinum analog [Pt(cis-dach)(DPPE) · $2NO_3$] for the evaluation of antimetastatic efficacy compared with two doses of cisplatinum. Unlike the untreated control group or the group treated with the low dose of cisplatinum, there were no metastases in either the high- or low-dose platinum analog-treated groups and the high-dose cisplatinum-treated group. The results obtained with this patient-like nude-mouse model of bladder cancer indicate that the new platinum analog appears to be a valuable lead compound with antimetastatic efficacy and clinical potential *(190)*.

Gemcitabine is a promising new agent that has recently been studied for palliation of advanced (stage IV) unresectable pancreatic cancer. We hypothesized that adjuvant gemcitabine would reduce recurrence and metastases following surgical resection of pancreatic cancer. To test this hypothesis, we evaluated gemcitabine on a GFP transductant of the human pancreatic cancer cell line BxPC-3 (BxPC-3-GFP) using SOI in mice. GFP allowed high-resolution fluorescence visualization of primary and metastatic growth. Five weeks after SOI, the mice were randomized into three groups. Group I received exploratory laparotomy only. Group II underwent surgical resection of the pancreatic tumor without further treatment. Group III underwent tumor resection followed by adjuvant treatment with gemcitabine, 100 mg/kg every 3 d for four doses, starting 2 d after resection. The mice were sacrificed at 13 wk following implantation, and the presence and location of recurrent tumor were recorded. Gemcitabine reduced the recurrence rate to 28.6% compared with 70.6% with resection only ($p = 0.02$) and reduced metastatic events 58% in the adjuvant group compared with resection only. This study, demonstrating that gemcitabine is effective as adjuvant chemotherapy post pancreatectomy, suggests a new indication of the drug clinically *(150)*.

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We determined the antitumor and antimetastatic efficacy of the camptothecin analog DX-8951f in an SOI metastatic mouse model of pancreatic cancer. DX-8951f showed efficacy against two human pancreatic tumor cell lines in this model. These cell lines were transduced with GFP, allowing high-resolution visualization of tumor and metastatic growth in vivo. The DX-8951f studies included both an early and advanced cancer model. In the early model, utilizing the human pancreatic cancer lines MIA-PaCa-2 and BxPC-3, treatment began when the orthotopic primary tumor was approximately 7 mm in diameter. DX-8951f was significantly effective against both MIA-PaCa-2 and BxPC-3 cells. In contrast, gemcitabine, the standard treatment for pancreatic cancer, did not have significant efficacy against MIA-PaCa-2 cells. Although gemcitabine showed significant activity against BxPC-3 primary tumor growth, it was not effective in metastasis. In the model of advanced disease, utilizing BxPC-3, treatment started when the orthotopic primary tumor was 13 mm in diameter. DX-8951f was significantly effective in a dose-response manner on the BxPC-3 primary tumor. DX-8951f also demonstrated antimetastatic activity in the late-stage model, significantly reducing the incidence of lymph node metastasis while eliminating lung metastasis. In contrast, gemcitabine was only moderately effective against the primary tumor and ineffective against metastasis at both sites in the late-stage model. Therefore, DX-8951f was highly effective against primary and metastatic growth in this very difficult-to-treat disease and showed significantly higher efficacy than gemcitabine, the standard treatment for pancreatic cancer. DX-8951f, therefore, has important clinical promise and has more positive features than the currently used camptothecin analog CPT-11, which requires metabolic activation and is toxic *(187)*.

The efficacy of recombinant human interferon-γ (rh IFN-γ) was evaluated for the treatment of human pleural adenocarcinoma in an SOI model of the human non-small cell lung cancer cell line H-460. IFN-γ was tested in three different dosages (25,000, 50,000, and 100,000 U) vs an untreated control through ip injection twice a day for 5 d, which was started 48 h after SOI. The results showed that IFN-γ can prolong the survival time of the tumor-bearing animals. The symptoms and signs of hypoxia, such as restricted physical activity and cyanosis owing to primary tumor growth in the thoracic cavity, as well as cachexia, developed much earlier in the control than in the IFN-γ-treated mice. The mice in the control group had succumbed by d 23 after tumor implantation. However, at that time 67% of the mice in the 100,000 U-treated group, 15% of the mice in the 50,000 U-treated group, and 16% of the mice in the 25,000 U-treated group were still alive. The orthotopically transplanted tumor grew rapidly and metastasized to the lung and liver in the untreated control. In the IFN-γ-treated groups, both primary tumor growth and metastasis were reduced, probably accounting for the increased survival rate. The results demonstrated dose-dependent efficacy of IFN-γ in suppressing symptomology, primary tumor growth, invasiveness, and metastasis of the human lung cancer cell line H 460, as well as increased survival of the tumor-bearing animals. These results suggest that clinical trials of IFN-γ should begin for treatment of pleural adenocarcinoma, for which there is no current effective therapy *(191)*.

We examined the importance of interleukine-8 receptor B (IL-8RB) mRNA expression in the growth of non-small cell lung cancer. Using the antisense oligonucleotide ICN 197, we were able to inhibit IL-8RB mRNA expression in vitro. The sequence-specific

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effect of antisense oligonucleotide and downregulation of IL-8RB mRNA was shown by reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot analysis. The proliferation of treated cells was measured by ^{3}H-thymidine incorporation. We found that treatment of these cells caused reversible growth inhibition and reversible downregulation of IL-8RB mRNA. Furthermore, we observed that treatment ~~with~~ of nude mice oligonucleotide ICN 197 inhibited the growth of tumors developed from non-small cell lung cancer cells injected subcutaneously. Our in vitro data suggest that IL-8RB mRNA expression is required to maintain the proliferative rate of these cells. Based on the data in vivo, oligonucleotide ICN 197 may be considered for the development of novel therapeutic treatment for lung cancer *(192)*.

~~Several synthetic inhibitors of MMPs show antitumor, antimetastasis, and anti-angiogenesis effects in various models. Synergistic effects of combinations with conventional cytotoxic agents have been reported.~~ The effects of a new selective MMP inhibitor, MMI-166, were evaluated on tumor growth, angiogenesis, and metastasis in an SOI liver metastatic model of human colon cancer (TK-4). We also investigated the synergistic effects of MMI-166 and a conventional cytotoxic agent, MMC, in this model. Mice transplanted orthotopically with TK-4 were divided into four groups: a control group treated with vehicle solution, an MMI-166 group in which MMI-166 was administered orally at a dose of 200 mg/kg, 6 d/wk for 5 wk, an MMC group in which MMC was administered intraperitoneally at a dose of 2 mg/kg/wk for 5 wk, and a combination group treated with MMI-166 and MMC. MMI-166 did not inhibit transplanted tumor growth but significantly inhibited liver metastasis compared with the control group and MMC group ($p < 0.01$). Significant antitumor and antimetastatic effects of the combination therapy were demonstrated. ~~The MVD detected by immunohistochemical staining with the ER-MP12 antibody tended to be lower in the MMI-166 and the combination groups.~~ These results suggest that MMI-166 has potential antimetastatic ability and a synergistic effect with MMC *(193,194)*.

~~Adriamycin® (ADM)~~ was encapsulated in a galactose-conjugated hepatotropic liposome (hLip-~~ADM~~), and its ability to enhance the antitumor effect while reducing toxicity was compared with that of free ~~ADM~~ and a control Lip-~~ADM~~ (cLip-~~ADM~~). An SOI model of human colon cancer xenograft TK-4 was used to induce liver metastases in mice. Liver metastasis occurred in 0/11 rats given hLip-~~ADM~~, whereas liver metastases developed in 10 of 12 mice in the control group and in 5 of 12 mice given cLip-~~ADM~~. Liposomal-~~ADM~~ did not have a significant inhibitory effect on transplanted tumor growth assessed 6 ~~wk~~ after transplantation. These findings indicate that hLip-~~ADM~~ may be an effective strategy for inhibiting liver metastases from human colon cancer *(195)*.

Tanaka et al. *(196)* established a mouse primary tumor resection model in which a transplanted tumor was resected after an SOI of colorectal cancer tissue to estimate the therapeutic effect of an angiogenesis inhibitor on metastasis. The angiogenesis inhibitor FR-118487 is a member of the fumagillin family. One mg/kg/d of FR-118487 was subcutaneously administered to nude mice for 1, 2, or 4 wk through an osmotic pump. Liver metastasis developed in seven of nine control mice, two of six mice that underwent the tumor resection 2 ~~wk~~ after transplantation (early resection), and in all seven of the mice that underwent the tumor resection 4 ~~wk~~ after transplantation (late resection). ~~In the short treatment trial, the~~ FR-118487 administration immediately after early resection completely inhibited both hepatic and peritoneal metastases, whereas its administration

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after late resection had no effect on liver metastasis. In the prolonged treatment trial, inhibitory effects of prolonged treatment with FR-118487 on both hepatic and peritoneal metastases after late resection were clearly demonstrated. The mice of the resection-alone group all died within 106 d after tumor inoculation, owing to metastases of colon carcinoma. In contrast, half of the mice that underwent resection and then received antiangiogenic therapy were alive at the end of the observation period (160 d after transplantation). In conclusion, the combination of surgery and subsequent antiangiogenic therapy may be useful to prevent the distant metastasis of colorectal cancer and to improve the prognosis of patients with colorectal cancer (196).

S-1 [1 *M* tegafur (FT)/0.4 *M* 5-chloro-2,4-dihydroxypyridine (CDHP)/1 *M* potassium oxonate (Oxo)], was developed as a new oral antineoplastic agent based on biochemical modulation of 5-FU by CDHP and Oxo. The therapeutic effect of S-1 on human colon cancer xenografts (TK-13) with high metastatic potential to the liver was evaluated. Small pieces of TK-13 were sutured into the cecal wall of 52 nude mice by SOI, and the animals were randomly divided into three groups [control ($n = 17$), UFT (combination of 1 *M* FT and 4 *M* uracil) ($n = 18$), and S-1 ($n = 17$)]. S-1 or UFT was administered orally at an equitoxic dose (S-1, 7.5 mg/kg; UFT, 17.5 mg/kg as FT) for 37 consecutive d beginning 10 d after the transplantation. S-1 showed higher tumor growth inhibition than UFT ($p < 0.05$) and also showed a significant antimetastatic effect on liver metastasis, whereas UFT did not. Liver metastasis developed in only 2 of the 17 mice (12%) in the S-1 group, whereas it developed in 9 of the 17 (53%) and 7 of the 18 (39%) in the control and UFT groups, respectively. Analysis of the area under the curve (AUC) revealed that S-1 yielded higher 5-FU levels in both tumor tissue (1.6 times) and plasma (2.5 times) than UFT. These results suggest that S-1 will show a higher clinical therapeutic effect against human colorectal cancer than UFT (197).

The efficacy of the combination of vascular endothelial growth factor neutralizing antibody (VEGFAb) and MMC was evaluated on MT-2, a human gastric cancer xenograft. When small pieces of MT-2 were transplanted by SOI into 62 nude mice, liver metastasis developed 6 wk after transplantation. The VEGFAb (100 μg/mouse) was administered ip in the VEGFAb group ($n = 14$) and the combination group ($n = 16$) twice a week from d 10 after transplantation. MMC (2 mg/kg) was administered in the MMC group ($n = 16$) and the combination group ($n = 16$) on d 10, 17, and 24 after transplantation. Compared with the control group, in which saline solution was administered ip, all three treatments inhibited tumor growth significantly, and the effects of MMC and combination therapy were potent. Liver metastases were also inhibited significantly by the administration of VEGFAb alone, MMC alone, or combination therapy. Liver metastasis developed in nine mice of the control group, three of the VEGFAb group, and four of the MMC group, but no mice had liver metastasis in the combination therapy group. However, a significant body weight loss and a decrease in spleen weight were observed in the MMC and combination groups, with no significant difference between the two groups. These results suggest that combination therapy with VEGFAb and MMC may be a potent therapy for human gastric cancer (198).

The therapeutic effect of VEGFAb on liver metastasis of an endocrine neoplasm was investigated. Cecal transplantation into nude mice of small pieces of EN-1, a xenotransplanted human intestinal endocrine neoplasm, resulted in liver metastasis. A treated group ($n = 19$) received 100 μg/mouse of VEGFAb intraperitoneally on alternate

days from d 10 after tumor transplantation, and the control group ($n = 19$) received saline. Five of the 19 control mice died of tumor progression, ~~of which 2 could not be evaluated~~. The cecal tumor weighed 6316 ± 2333 mg ($n = 17$) in the control group and 1209 ± 837 mg ($n = 19$) in the treated group ($p < 0.01$) 6 ~~wk~~ after transplantation. Liver metastasis developed in 16 of 17 control mice and in 2 of 19 treated mice ($p < 0.01$). The VEGF level of the whole cecal tumor in the control group was significantly higher than that in the treated group (305.1 ± 174.1 vs 54.7 ± 41.2 mg; $p < 0.001$). VEGFAb did not cause any body weight loss (28.52 ± 1.63 in the control vs 28.44 ± 1.71 g in the treated group). These results indicate that VEGFAb may be a novel therapeutic agent for endocrine neoplasm with distant metastasis *(199)*.

To evaluate VEGFAb further, four human carcinoma xenografts, two human colon carcinomas (TK4 and TK 13), and two gastric carcinomas (MT2 and MT5) were transplanted by SOI into nude mice. The anti-VEGF antibody (~~MV833~~, 100 μg/mouse) or the same volume of saline was administered ip on alternative days from ~~d 10~~ after transplantation. With each of the four xenografts, administration of ~~MV833~~ significantly inhibited not only primary tumor growth but also macroscopic liver metastasis, although the growth rate varied. The inhibitory effect of ~~MV833~~ on primary tumor growth appeared to have no correlation with the level of VEGF in the tumor. Body weight gain in each treated group was comparable to that in the control group. No toxicity of the antibody was observed. These results suggest that an anti-VEGF antibody can be effective against a wide variety of cancers and that VEGF may be a possible target for cancer therapy *(200)*.

The effect of an angiogenesis inhibitor, TNP-470, on primary tumor growth, liver metastasis, and peritoneal dissemination of gastric cancer was investigated ~~by means of~~ ~~an~~ SOI model of two human gastric cancers, MT-2 and MT-5. TNP-470 showed a significant inhibitory effect on the growth of primary tumors after orthotopic transplantation of both xenografts when given at a dose of 30 mg/kg on alternate days from ~~d~~ 7 after transplantation ~~(early treatment)~~. However, growth of the MT-2 primary tumor was not inhibited by administration from ~~d 14~~ after transplantation ~~(late treatment)~~. Liver metastasis was significantly prevented by early treatment of TNP-470. In particular, early treatment of MT-2 completely inhibited the development of macroscopic foci in the liver and was significantly more effective than late treatment. Peritoneal dissemination was also inhibited. ~~Thus, TNP-470 was revealed to have strong inhibitory activity not only on primary tumors and liver metastases but also against peritoneal dissemination. These results suggest that this agent may provide a new approach to the treatment of gastric cancer *(201)*.~~

The colon tumor xenograft TK-4 was transplanted by SOI and treated with TNP-470. Treatment was with 30 mg/kg of TNP-470 on alternate days from ~~d 10~~ after transplantation. The rate of hepatic metastases from orthotopically transplanted tumors of five strains was 38–79%. ~~Interestingly~~, TK-4 has *K-ras* and *p53* mutations, and ~~overexpression~~ of p53 ~~protein~~ induced hepatic metastases from both orthotopic (79%) and subcutaneous tumors (44%). Although TNP-470 only significantly inhibited subcutaneous tumor growth, its antimetastatic effect was significantly demonstrated on the hepatic metastases *(202)*. ~~*p53* mutation is thought to enhance angiogenesis, favoring the growth of the hepatic metastases. TNP-470 had antimetastatic efficacy on TK-4 for hepatic metastases *(202)*.~~

The antimetastatic effect of TNP-470 was investigated further in nude mice in SOI models with human colon cancer. ~~Small pieces of~~ tumors from three established human colon cancer cell lines (TK-3, TK-4, and TK-9), which were maintained in nude mice,

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were implanted into the cecal wall of nude mice via a small incision in the serosa. TNP-470 (20 or 30 mg/kg) was given sc every other day from ~~d 10~~ after implantation, and the mice were sacrificed after 6 ~~wk~~. There was no difference in the weight of the implanted tumors (control group: 0.45 ± 0.29 g vs treated group: 0.49 ± 0.27 g). An antimetastatic effect of TNP-470 was clearly demonstrated in a dose-dependent manner. In the mice given 20 mg/kg TNP-470, liver metastasis developed in 3 of 10 cases. In the 30-mg/kg group, metastasis developed in only 1 of 17 mice, whereas it developed in 22 of 32 mice of the control group. The number of metastatic foci was significantly less in the treated groups. TNP-470 ~~effectively~~ prevented liver metastasis, but had no effect on the growth of the primary tumor. ~~These results indicate that the angiogenesis inhibitor TNP-470 has a strong inhibitory activity against in vivo hepatic metastasis of human colon cancer (203).~~

The antitumor and antimetastatic efficacy of TNP-470 and MMC, ~~a representative antineoplastic agent~~, were investigated in ~~an~~ SOI model of human colon cancer, TK-4. Mice were randomly divided into three groups; a control group given saline solution, a group receiving TNP-470 and a group receiving MMC. TNP-470 was given subcutaneously on alternate days for 5 ~~wk~~ from ~~d 10~~ after cecal transplantation, and MMC was administered intraperitoneally once a week from d 10 after cecal transplantation. MMC significantly inhibited cecal tumor growth. In the control group, liver metastases developed in 9 of 10 mice, including 3 with more than 20 metastatic foci. Liver metastasis also developed in 8 of 10 mice receiving MMC, 2 of which had many metastases. In contrast, liver metastasis developed in only two of eight mice in the TNP-470 group, and neither of these animals had numerous metastases *(204)*.

9. NEW DIRECTIONS

9.1. Dual-Color Imaging of Tumor-Host Interaction

We have established a dual-color fluorescence imaging model of tumor-host interaction based on a red fluorescent protein (RFP)-expressing tumor growing in GFP transgenic mice. This model allowed visualization of the tumor-stroma interaction including tumor angiogenesis and infiltration of lymphocytes in the tumor. Transgenic mice, expressing the GFP under the control of a chicken β-actin promoter and cytomegalovirus enhancer, were used as the host. All the tissues from this transgenic line, with the exception of erythrocytes and hair, fluoresce green under blue excitation light. B16F0 mouse melanoma cells were transduced with pLNCX2-DsRed-2-RFP plasmid. The B16F0-RFP tumor and GFP-expressing stroma could be clearly imaged simultaneously in excised tissue. Dual-color imaging allowed resolution of the tumor cells and the host tissues down to the single cell level. Tumor stroma included fibroblast cells, tumor-infiltrating lymphocytes, blood vessels, and capillaries, all expressing GFP. GFP stromal cells were readily distinguished from the RFP-expressing tumor cells. This dual-color fluorescence imaging system should facilitate studies for understanding tumor-host interaction during tumor growth and tumor angiogenesis. The dual-color system also provides a powerful tool to analyze and isolate tumor-infiltrating lymphocytes and other host stromal cells interacting with the tumor for therapeutic and diagnostic/analytic purposes *(208)*.

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